

Large-scale serum protein biomarker discovery in Duchenne muscular dystrophy

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Serum biomarkers in Duchenne muscular dystrophy (DMD) may provide deeper insights into disease pathogenesis, suggest new therapeutic approaches, serve as acute read-outs of drug effects, and be useful as surrogate outcome measures to predict later clinical benefit. In this study a large-scale biomarker discovery was performed on serum samples from patients with DMD and age-matched healthy volunteers using a modified aptamer-based proteomics technology. Levels of 1,125 proteins were quantified in serum samples from two independent DMD cohorts: cohort 1 (The Parent Project Muscular Dystrophy–Cincinnati Children's Hospital Medical Center), 42 patients with DMD and 28 age-matched normal volunteers; and cohort 2 (The Cooperative International Neuromuscular Research Group, Duchenne Natural History Study), 51 patients with DMD and 17 age-matched normal volunteers. Forty-four proteins showed significant differences that were consistent in both cohorts when comparing DMD patients and healthy volunteers at a 1% false-discovery rate, a large number of significant protein changes for such a small study. These biomarkers can be classified by known cellular processes and by age-dependent changes in protein concentration. Our findings demonstrate both the utility of this unbiased biomarker discovery approach and suggest potential new diagnostic and therapeutic avenues for ameliorating the burden of DMD and, we hope, other rare and devastating diseases.

proteomics | muscular dystrophy | biomarkers | SOMascan | SOMAmer

There is an urgent need for a reliable surrogate biomarker or set of biomarkers for Duchenne muscular dystrophy (DMD), ideally based on readily accessible and measurable molecules (1). DMD is a severe form of myopathy with an incidence of about 1 in 3,600–9,337 boys worldwide (2, 3), and is a result of different types of mutations in the X-linked *DMD* gene that abolish the expression and biological activity of dystrophin, an essential protein for muscle-fiber plasma membrane integrity and myofiber function (4, 5). Clinically, the disease is characterized by progressive muscle wasting, leading to loss of ambulation by 8–15 y of age and early death from complications from respiratory, orthopedic, and cardiac problems (2, 6).

Several current drug-development programs are focused on slowing or preventing the progressive muscle loss in DMD either in conjunction with the standard of care treatment or as stand-alone therapies. Standard of care is currently chronic high-dose glucocorticoids, which are able to prolong ambulation by 3–4 y (7, 8) and slow disease progression, but are associated with a significant array of side effects (2, 6, 9, 10). Promising therapeutic approaches for DMD include restoring expression of the dystrophin gene via exon-skipping strategies (11–13), viral-based gene therapies (14, 15), and nonsense suppression/read-through strategies (16). Other genetic approaches include delivering

minidystrophins, up-regulation of utrophin to compensate for the missing dystrophin, and many others (17). Pharmacological strategies in development include dissociative steroid drugs, which offer the potential of greater efficacy and lesser side effects (18), other anti-inflammatory therapies, and effectors of signaling pathways (19). The current primary clinical endpoint used for determining efficacy in the majority of these therapeutic approaches for ambulatory boys with DMD is the “six-minute walk test” (20, 21), although it is not ideal (22).

Blood provides a circulating protein representation of all body tissue in both normal and pathological conditions, and serum proteins are emerging as useful biomarkers for diagnosis and prognosis of a growing number of diseases (23, 24). Mass spectrometry (MS)-based proteomic screens recently have proved successful at de novo biomarker identification in DMD (25). However, verification and validation of MS-discovered serum biomarkers remain challenging (24). Other approaches, such as multiplexed antibody or aptamer-based assays, are being considered for proteome screens because of their potential for higher throughput and better sensitivity, which may help overcome the validation challenges of identified biomarkers. For example, a

Significance

Duchenne muscular dystrophy (DMD) is a rare and devastating muscle disease caused by mutations in the X-linked *DMD* gene (which encodes the dystrophin protein). Serum biomarkers hold significant potential as objective phenotypic measures of DMD disease state, as well as potential measures of pharmacological effects of and response to therapeutic interventions. Here we describe a proteomics approach to determine serum levels of 1,125 proteins in 93 DMD patients and 45 controls. The study identified 44 biomarkers that differed significantly between patients and controls. These data are being made available to DMD researchers and clinicians to accelerate the search for new diagnostic, prognostic, and therapeutic approaches.

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Conflict of interest statement: L.G. is the founder and a stakeholder in SomaLogic, Inc.; E.B., R.K.D., B.M., M.N., B.S., F.S., D.S., and S.W. are employees and stakeholders in SomaLogic, Inc.; Y.M.K. has been affiliated with the Eli Lilly and Co. since October 2012.

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recent study using an antibody-based array against 384 target proteins identified 11 protein biomarkers of disease across different muscular dystrophies from patient samples gathered from four different clinical sites (26). In addition, a modified aptamer-based technology (the SOMAscan assay) is emerging as another highly sensitive and multiplexed assay for biomarker discovery and validation (27–29). Based on novel reagents (Slow Off-rate Modified Aptamers, or SOMAmer reagents) that recognize specific conformational epitopes of native 3D proteins with high specificity and high sensitivity (30–32), the SOMAscan assay measures levels of 1,125 analytes in only 65 μ L of serum over a wide dynamic range (>8 logs of concentration). Because the SOMAscan assay relies on the availability of the protein epitopes (i.e., the epitopes are not blocked by other protein binding, posttranslational modifications, and so forth), what is measured in the assay and the actual protein concentration in the sample being interrogated is frequently but not always correlated. In the same manner, ELISAs for the same proteins also are frequently but not always correlated.

Because blood is the preferred diagnostic clinical material, and biomarkers in the blood can differ by several orders-of-magnitude in abundance, the SOMAscan assay may be a path forward to identify and verify key blood-based biomarkers for DMD and other diseases. We used the SOMAscan technology to screen for protein biomarkers associated with DMD using serum samples from two independent cohorts collected in different locations and run at different times (cohort information in *Demographics, Characteristics, and Enrollment Criteria of the PPMD-C and CINRG Cohorts* and *Dataset S1*). The first cohort analyzed was from The Parent Project Muscular Dystrophy–Cincinnati Children’s Hospital Medical Center (hereafter PPMD-C), which included the goal of identifying alternative treatment paths (i.e., nondystrophin-centric) for patients with DMD. The second cohort analyzed was from The Cooperative International Neuromuscular Research Group, Duchenne Natural History Study (hereafter CINRG) (33), which included the goal of identifying changes in biomarkers with age in patients with DMD. In the present study, we compared the data from these two independent studies. This process enabled us to identify 44 biomarkers in the blood associated with DMD: 24 that are significantly increased and 20 that are significantly decreased in patients with DMD.

These data suggest new protein targets and biomarkers for further DMD studies. The data also may facilitate future clinical studies designed to identify new therapeutics for DMD, as well as further demonstrating the utility of the SOMAscan assay technology for identifying protein biomarkers for both rare and common diseases. We are making our data fully available to the DMD research community to enable further studies that may be suggested by these findings.

Results

Independent SOMAscan Assay Analyses on Two DMD Cohorts. Two independent DMD natural history cohorts were used in this study. The PPMD-C cohort comprised 42 DMD patients (2–27 y old) and 28 healthy male volunteers (4–28 y old, most often from the DMD male sibling pool). The CINRG cohort comprised 51 DMD patients (age range 4–29 y old) and 17 healthy male volunteers (age range, 6–18 y old). The demographics, characteristics, and enrollment criteria of the two cohorts are summarized in *Demographics, Characteristics, and Enrollment Criteria of the PPMD-C and CINRG Cohorts* and *Dataset S1*. In the initial analysis, the PPMD-C study design included steroid treatment for a subset of patients and the CINRG study included ambulatory status. Steroid treatment had no statistically significant effect on the 44 protein biomarkers described below, and ambulatory status was relevant only insofar as it related to increasing age but had no statistically significant effect on the

results. Our standard quality-control protocols detected no significant difference in the samples from the two cohorts.

Serum samples were tested using the SOMAscan protein biomarker discovery assay (SomaLogic), which detects 1,125 proteins simultaneously using 65 μ L of serum. At a 1% false-discovery rate (FDR) (*Materials and Methods*), based on SOMAscan assay data from a total of 93 DMD patients and 45 age-matched controls from the two cohorts, we identified 44 proteins that consistently differed in the serum in both cohorts when comparing DMD patients vs. controls. The UniProt names and a measure of differential expression [the signed Kolmogorov–Smirnov (KS) distance] for these 44 proteins in each cohort are shown in Table 1, along with an indicator of each protein’s known enrichment in muscle tissue. The entire 1,125 protein SOMAscan assay results for each cohort independently are listed in *Dataset S2*.

Of the 44 protein biomarkers that were significantly different between DMD and controls, detected levels increased for 24 and decreased in 20 in DMD patients compared with normal controls. Fig. 1 shows the empirical cumulative distribution functions (CDFs) for six representative proteins from the combined cohort analysis [three proteins that are increased are troponin 1 fast skeletal muscle (TNNI2), myoglobin (MB), heat-shock protein 70 (HSPA1A); and three that are decreased are proto-oncogene tyrosine-protein kinase receptor Ret (RET), gelsolin (GSN), bone sialoprotein 2 (IBSP) in DMD patients vs. controls]. These

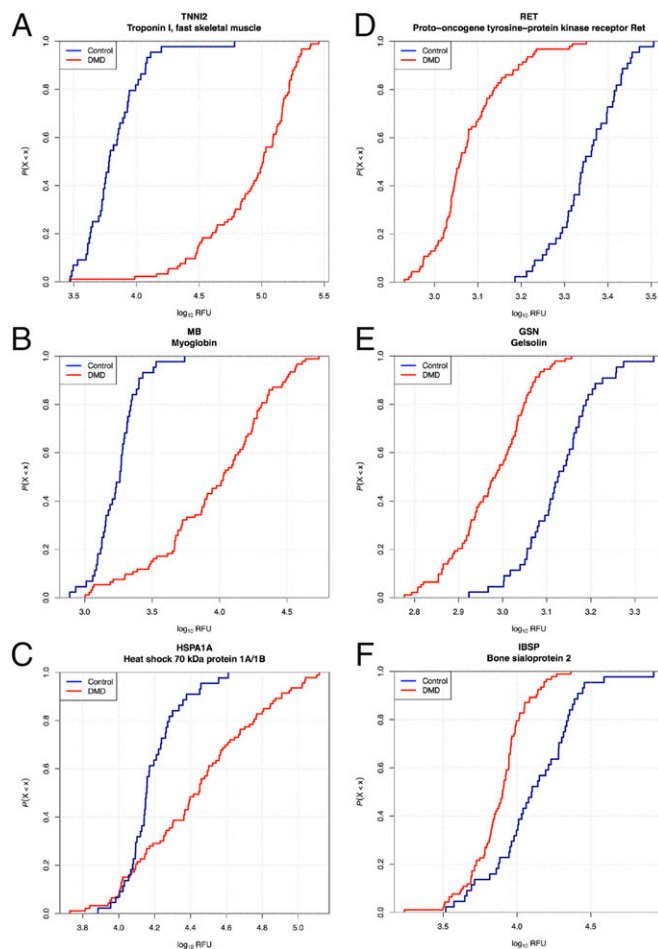


Fig. 1. Representative CDFs of proteins that are up or down in DMD patients vs. controls from both cohorts. Up proteins: (A) Troponin I, fast skeletal muscle, (B) myoglobin, (C) heat-shock protein 70. Down proteins: (D) RET, (E) gelsolin, (F) bone sialoprotein 2.

Correlation Between Biomarker Levels and Age of DMD Patients. In this DMD study, age is a proxy for disease severity, because older patients have more advanced disease. Because multiple biological samples over time from individual patients were not available, we

Protein name (UniProt)	Gene name (UniProt)	PPMD-C signed KS distance	CINRG signed KS distance	Average KS	Rank	Muscle enriched	Age-related change group no.
Troponin I, fast skeletal muscle	<i>TNNI2</i>	1.000	0.918	0.959	1	Yes	1
Carbonic anhydrase 3	<i>CA3</i>	0.964	0.938	0.951	2	Yes	1
Fatty acid-binding protein, heart	<i>FABP3</i>	1.000	0.882	0.941	3	Yes	1
Troponin I, cardiac muscle	<i>TNNI3</i>	0.917	0.961	0.939	4	Yes	1
Creatine kinase M-type	<i>CKM</i>	0.976	0.839	0.908	5	Yes	1
Mitogen-activated protein kinase 12	<i>MAPK12</i>	1.000	0.797	0.898	6	Yes	1
Alanine aminotransferase 1	<i>GPT</i>	0.738	0.941	0.840	7	No	1
Myoglobin	<i>MB</i>	0.857	0.820	0.838	8	Yes	1
Fibrinogen	<i>FGA FGB FGG</i>	0.810	0.784	0.797	9	No	1
Phospholipase A2, membrane associated	<i>PLA2G2A</i>	0.762	0.800	0.781	10	No	3
Acidic leucine-rich nuclear phosphoprotein 32 family member B	<i>ANP32B</i>	0.821	0.706	0.764	11	No	1
Hepatoma-derived growth factor-related protein 2	<i>HDGFRP2</i>	0.738	0.691	0.715	12	No	3
40S ribosomal protein S7	<i>RPS7</i>	0.690	0.734	0.712	13	No	1
Glucose-6-phosphate isomerase	<i>GPI</i>	0.774	0.604	0.689	14	Yes	1
Heparin cofactor 2	<i>SERPIND1</i>	0.560	0.813	0.686	15	No	3
Persephin	<i>PSPN</i>	0.595	0.757	0.676	16	No	3
Calcium/calmodulin-dependent protein kinase II α	<i>CAMK2A</i>	0.738	0.586	0.662	17	Yes	1
Malate dehydrogenase, cytoplasmic	<i>MDH1</i>	0.595	0.706	0.651	18	Yes	1
L-lactate dehydrogenase B chain	<i>LDHB</i>	0.631	0.608	0.619	19	Yes	1
Aminoacylase-1	<i>ACY1</i>	0.643	0.577	0.610	20	No	1
Proteasome subunit α type-2	<i>PSMA2</i>	0.571	0.600	0.586	21	No	3
C-X-C motif chemokine 10	<i>CXCL10</i>	0.560	0.600	0.580	22	No	3
cAMP-dependent protein kinase catalytic subunit α	<i>PRKACA</i>	0.560	0.570	0.565	23	No	1
Heat-shock 70 kDa protein 1A/1B	<i>HSPA1A</i>	0.476	0.600	0.538	24	Yes	1
Proto-oncogene tyrosine-protein kinase receptor Ret	<i>RET</i>	-0.917	-0.961	-0.939	1	No	2
Growth/differentiation factor 11	<i>GDF11</i>	-0.667	-0.941	-0.804	2	No	4
Complement decay-accelerating factor	<i>CD55</i>	-0.762	-0.745	-0.754	3	No	4
Cadherin-5	<i>CDH5</i>	-0.821	-0.675	-0.748	4	No	2
Tumor necrosis factor receptor superfamily member 19L	<i>RELTL</i>	-0.786	-0.706	-0.746	5	No	4
Gelsolin	<i>GSN</i>	-0.750	-0.718	-0.734	6	Yes	4
Wnt inhibitory factor 1	<i>WIF1</i>	-0.679	-0.714	-0.697	7	No	2
Contactin-5	<i>CNTN5</i>	-0.655	-0.702	-0.678	8	No	2
Prolyl endopeptidase FAP	<i>FAP</i>	-0.643	-0.659	-0.651	9	No	2
Jagged-1	<i>JAG1</i>	-0.679	-0.613	-0.646	10	No	2
Netrin receptor UNC5C	<i>UNC5C</i>	-0.560	-0.718	-0.639	11	No	2
Kunitz-type protease inhibitor 1	<i>SPINT1</i>	-0.667	-0.597	-0.632	12	No	2
Protein SET	<i>SET</i>	-0.500	-0.722	-0.611	13	No	2
Disintegrin & metalloproteinase domain-containing protein 9	<i>ADAM9</i>	-0.595	-0.600	-0.598	14	No	2
Cell adhesion molecule L1-like	<i>CHL1</i>	-0.583	-0.589	-0.586	15	No	2
Osteomodulin	<i>OMD</i>	-0.452	-0.718	-0.585	16	No	2
WAP, Kazal, Ig, Kunitz and NTR domain-containing protein 1	<i>WFIKK1</i>	-0.464	-0.699	-0.581	17	No	4
Bone sialoprotein 2	<i>IBSP</i>	-0.476	-0.613	-0.544	18	No	2
Interleukin-34	<i>IL34</i>	-0.488	-0.558	-0.523	19	No	2
Neurogenic locus notch homolog protein 3	<i>NOTCH3</i>	-0.488	-0.550	-0.519	20	No	2

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instead examined the age-dependence in protein levels across the whole cohort. Proteins were screened using a single protein linear regression model to identify candidates where patient age was a useful predictor of protein concentration. We identified four general groupings of differential protein changes for the 44 biomarkers identified in this study (Fig. 2, Table 1, and Fig. S2).

Group 1 has protein biomarkers that were at their highest levels in young patients with DMD—far higher than in normal controls—and then decreased as a function of age in DMD while remaining relatively unchanged or increasing slightly with age in controls (18 proteins, represented by creatine kinase) (Fig. 2A).

Group 2 has proteins that changed with age in DMD and controls, but which were significantly lower in patients at most ages (15 proteins, represented by RET) (Fig. 2B).

Group 3 has protein biomarkers that changed with age in DMD and controls, but which were significantly higher in patients at most ages (six proteins, represented by phospholipase A2) (Fig. 2C).

Group 4 has protein biomarkers whose concentrations were very similar between DMD and controls at an early age, but then decreased with age in DMD patients while increasing in controls [five proteins, represented by growth differentiation factor 11 (GDF11)] (Fig. 2D).

Age-related regression plots for all 44 proteins are available in Fig. S2.

Discussion

Using the SOMAscan assay, we identified 44 circulating serum biomarkers associated with DMD patients vs. healthy controls from two independent cohorts with a 1% FDR-corrected significance level. Although some of us are experts in this field, in the following discussion we have tried to minimize hypothesizing about the potential meaning of the markers discovered in this study so as to provide the wider DMD community an unbiased opportunity to pursue these results following their own interpretations.

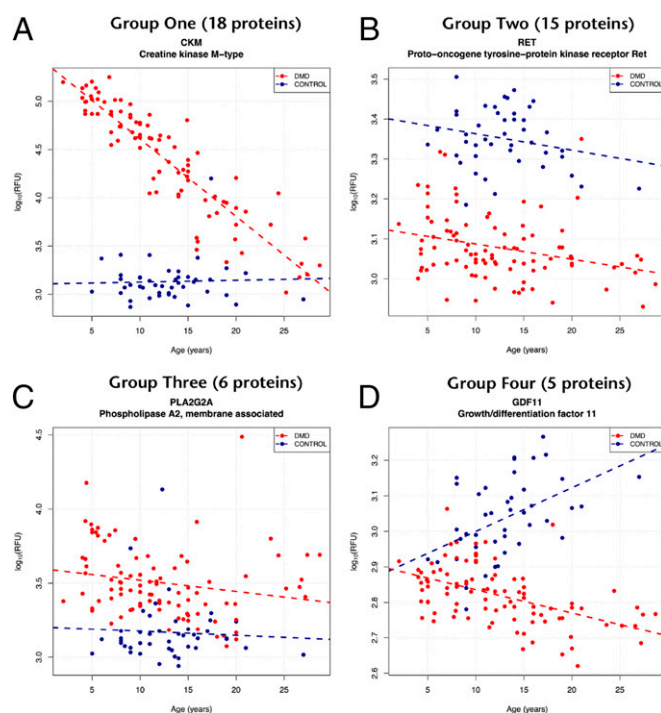


Fig. 2. Example proteins from the four “types” of age-related changes in protein signal levels seen in DMD patients (red) vs. controls (blue) from both cohorts. (A) Group 1, creatine kinase; (B) group 2, RET; (C) group 3, phospholipase A2; (D) group 4, growth-differentiation factor 11.

The most striking differences between DMD patients and controls were observed in the young age range (4–10 y old), where the most significant biomarkers were elevated up to two orders-of-magnitude in serum samples of DMD patients relative to healthy volunteers (group 1 proteins). These biomarkers then declined with age and disease progression. These “creatine kinase-like” proteins (Fig. 2A) are mostly of muscle origin and their early elevation in blood is likely associated with muscle damage/cell death and inflammation at an early age, and their subsequent decline with age is most likely the result of loss of muscle mass in the DMD patients.

The high-to-low change in concentration of these creatine kinase-like proteins likely reflects high myofiber membrane instability/damage, necrosis, and leakage of cytoplasm into the extracellular space. This group includes muscle-enriched proteins such as creatine kinase M-type (CK-M) itself, fatty acid binding protein 3 (FABP3), myoglobin (MB), carbonic anhydrase III (CA3), malate dehydrogenase (MDH1), lactate dehydrogenase B (LDHB), glucose phosphate isomerase (GPI), Hsp70 (HSPA1A), troponin I, fast skeletal muscle (TNNI2), troponin I, cardiac muscle (TNNI3), mitogen-activated protein kinase 12 (MAPK12), and calcium-calmodulin-dependent protein kinase II α (CAMK2A). Most of these muscle leakage proteins have been previously reported by others to be elevated in DMD boys relative to healthy volunteers (25, 26), except for Hsp70, MAPK12, and CAMK2A, which are novel to this study.

We also identified several proteins (all group 2) that are associated with connective tissue remodeling, including prolyl endopeptidase FAP (FAP), protein jagged-1 (JAG1), bone sialoprotein 2 (IBSP), ADAM metalloproteinase domain 9 (ADAM9), cadherin-5 (CDH5), neural cell adhesion molecule L1-like protein (CHL1), osteomodulin (OMD), and contactin-5 (CNTN5). Each of these proteins was found to be significantly lower in DMD patients than in controls at all ages. These proteins may regulate connective tissue remodeling in skeletal muscle.

Several other proteins identified in this study are functionally associated with inflammation and innate immune pathways, including: group 2 protein interleukin-34 (IL-34); group 3 proteins C-X-C motif chemokine 10 (CXCL10), phospholipase A2 (PLA2G2A), and hepatoma-derived growth factor-related protein 2 (HDGFRP2); and group 4 proteins CD55/complement decay-accelerating factor (CD55) and RELT tumor necrosis factor receptor (RELT). These proteins do not show significant change as a function of age, with the two exceptions of CD55 (decreases with age in DMD and increases with age in controls) and fibrinogen (increases with age in both DMD and controls). Two of the above group 3 proteins (PLA2G2A and CXCL10) are of particular interest because they could be useful pharmacodynamic biomarkers to monitor efficacy of anti-inflammatory agents in DMD patients. Phospholipase A2 activity has been reported to be dramatically increased (10-fold) in the skeletal muscle of DMD patients relative to controls and is associated with muscle inflammation (34), consistent with the high serum levels reported here. CXCL10 is an extracellular chemokine and its elevation in serum could be associated with increased T-cell infiltration in inflamed skeletal muscle (35).

Another intriguing protein that emerged from our studies is the group 3 protein persephin, a member of the GDNF family of neurotrophic factors. Persephin signals through the RET receptor tyrosine kinase-mitogen-activated protein kinase pathway, and is known to be expressed in skeletal muscle, motor neurons and, perhaps, Schwann cells (26). Although its role in motor neurons is uncertain, persephin may be involved in the reinnervation process, as it has been observed to stimulate neurite outgrowth in oculomotor neurons (36). Thus, the increased detection of persephin and decreased detection of RET (group 2) levels in DMD patients vs. controls (Table 1) could be a marker of the ongoing denervation/reinnervation that is occurring. In terms

positive test statistic indicating higher protein levels in DMD patients than in controls. We show the empirical CDF of the protein levels as an accurate representation of the underlying signals in the two patient populations. In all cases the ordinant represents the fraction of patients with signal levels below the corresponding abscissa reported in \log_{10} RFU. In statistical tests we account for multiple comparisons by reporting the FDR computed using the BH method (43) in the *p.adjust* function in the R base package, *stats* (44). All statistical analysis performed with the R language for statistical computing v3.1.2 (2014-10-31).

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